(51) International Patent Classification 5 :	A1	(11) International Publication Number:	WO 93/14108
C07K 1/00, C12Q 1/68 G01N 33/68 // C07K 15/04		(43) International Publication Date:	22 July 1993 (22.07.93)

(54) Title: SELECTION OF BINDING-MOLECULES

(57) Abstract

Methods of designing and producing sequence-specific DNA binding proteins, methods of determining the affinity of a specific binding molecule for a target and products produced by these methods are disclosed. The methods include: forming a reversible bond between a specific binding molecule and the target and determining the susceptibility of the reversible bond to reversal as a measure of the affinity of the binding molecule for the target.

SELECTION OF BINDING-MOLECULES

Description

Background of the Invention

Small molecules which bind to other molecules with
5 specific affinity are important in many biological pro-
cesses.   The importance of sequence specific DNA-binding
proteins in biology became apparent in the 1960's with
the establishment of models for gene regulation.   Because
of their important roles, it would be useful to be able
10 to design small molecules which can mimic or replace
naturally-occurring molecules.   However, despite consid-
erable interest in the design and production of small
binding molecules, a rational process for the design,
synthesis and selection of such molecules has not yet
15 been developed.

Summary of the Invention

The present invention relates to methods of design-
ing and producing a member of a binding pair which spe-
cifically binds to its partner.   It further relates to
20 the products resulting from the methods.   Such members
are referred to herein as specific binding molecules.   It
particularly relates to designing and synthesizing mole-
cules which specifically bind a desired target, such as a
DNA sequence; these molecules are referred to as se-
25 quence-specific DNA binding molecules and are also the
subject matter of the present invention.   Molecules, such
as the sequence-specific binding molecules (also referred
to herein as specific binding molecules) designed by the
present method can be a peptide (D-, L- or a mixture of
30 D- and L-), a peptidomimetic, a complex carbohydrate or
other oligomer of individual units or monomers which

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binds specifically to its binding partner (e.g., to DNA).
The present invention further relates to molecules,
particularly sequence-specific DNA molecules, designed
and produced by the present method and to uses therefor.
5 Specific binding molecules produced by the present method
can be used in any application in which predictable or
specific joining of two members of a binding pair is
desired.

    In one embodiment, sequence-specific DNA binding
10 molecules produced by the methods described herein, are
useful as gene regulatory molecules, such as molecules
which mimic the tight and specific DNA binding character-
istics of transcription factors, which play important
roles in regulation of gene transcription by increasing
15 or decreasing the rate of mRNA synthesis. Most commonly,
genes are regulated at the level of transcription by
proteins, referred to as transcription factors, which
bind promoter DNA. A critical step in gene regulation by
transcription factors is binding a factor to its specif-
20 ic, or target, DNA sequences in the promoter. Sequence-
specific DNA binding molecules designed and produced by
the present method can be used as molecules which mimic
the tight and specific DNA binding characteristics of
transcription factors and, as a result, exert control
25 over gene expression. Sequence specific DNA binding
molecules can be used, for example, to control (enhance
or repress) gene expression in vivo and, thus, serve as
the basis for development of new therapeutic strategies
for treating diseases or conditions in which there is a
30 genetic defect. For example, a sequence-specific DNA
binding molecule of the present invention can be used as
an artificial or synthetic transcription repressor which
is designed to bind a particular promoter and inhibit
transcription of the gene under its control. An artifi-
35 cial or synthetic transcription repressor can be used to

inhibit expression of a gene whose over-expression is associated with a disease or condition. Genetic diseases showing dominant inheritance, such as Huntington's disease, are promising candidates for counteraction by

5 transcriptional inhibitors designed and produced by the method of the present invention.

The present method of designing and producing a sequence-specific binding molecule is exemplified herein by the method of designing and producing a sequence-

10 specific DNA binding molecule, particularly, a sequence-specific DNA binding peptide. In the present method of designing and producing a sequence-specific DNA binding peptide, the following steps are carried out:

A desired or target molecule (e.g., a desired or

15 target DNA sequence, or molecule) is synthesized or otherwise provided, which contains a first moiety capable of forming a reversible bond with a second moiety. The target DNA sequence is one for which a sequence specific binding molecule, particularly a sequence specific DNA

20 binding peptide, is to be designed and produced. The target DNA sequence is combined with a test-binding molecule, which contains a moiety capable of forming a reversible bond with the moiety present on the target sequence, such as the target DNA sequence. The test-

25 binding molecule (also referred to herein as test-molecule) comprises a unit such as an amino acid residue, to be assessed for its ability to bind to the desired DNA sequence. The resulting combination of target DNA sequences and test-molecules is maintained under conditions

30 that are appropriate for the formation of a reversible bond between the first moiety (i.e., on the DNA sequence) and the second moiety (i.e., on the test-molecule) and binding of the unit being assessed to a region of the target sequence. Thus, under the appropriate conditions,

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DNA sequence-test-binding molecule complexes are formed,
or produced.

   These complexes are then subjected to conditions
under which the reversible bond between the moiety on the
5 DNA sequence and the moiety on the test-molecule is
reversed (i.e, disrupted or broken). Under a set of
specified conditions, if the unit of the test-molecule is
bound tightly to the DNA sequence (i.e., in a site-spe-
cific manner) the test-molecule will remain bound to, or
10 associated with, the desired DNA sequence. However, if
the unit of the test-molecule is weakly bound to the DNA
sequence, under the same specified conditions, the test-
molecule will easily dissociate from the desired DNA se-
quence. Thus, a mixture is produced which contains
15 complexes of the test-molecule bound to the desired
target sequence, uncomplexed target molecules and
uncomplexed test-molecules. In the case in which a
sequence-specific DNA-binding molecule (e.g., a DNA
binding peptide) is being produced, the resulting mixture
20 contains complexes, uncomplexed target DNA sequence and
uncomplexed test molecules.

   The identity of the test-molecule present in the
complexes, and the order of the units comprising the
test-molecule, is determined by the present method by
25 carrying out the above-described process. The process is
carried out a sufficient number of times to identify a
binding partner, such as a DNA binding protein, of appro-
priate makeup and sufficient length to bind to the target
DNA and remain bound to the DNA, and subsequently deter-
30 mining the identity and order of the units (e.g., amino
acid residues) in the binding partner produced. With
each subsequent cycle, the test-molecule includes one
more unit to be assessed than the test-molecule of the
previous cycle; the test-molecule in the complex which is
35 formed also has one additional unit than the complex in

the previous cycle. Thus, following the method described
herein, a sequence-specific DNA binding molecule is
designed and produced.

In a preferred embodiment, the moiety present on the
5 target DNA and on the target molecule is a thiol group,
the reversible bond formed between the two moieties is a
disulfide bond, the test-molecule is a peptide and the
unit to be assessed is an amino acid residue. In this
embodiment, a DNA molecule of a desired sequence which
10 contains a thiol group attached at a specific site on the
sequence is combined with a synthetic peptide which also
contains a thiol group. The peptide has the formula
CO_2H-Cys-Xaa-NH_2. The DNA molecule and the peptide bind,
or associate, via the formation of a reversible disulfide
15 bond, thus, forming a DNA-peptide complex.

In another embodiment, a mixture of peptides can be
used, all of which have the formula CO_2H-Cys-Xaa-NH_2 and
each of which differs in the amino acid residue Xaa (Xaa
can be any amino acid residue which lacks an -SH group).
20 In either embodiment, each peptide will have a different
association constant for the DNA sequence, and these
differences will affect the reversibility, or reducibili-
ty, of the disulfide bond.

Under reversing conditions, such as subjecting the
25 formed complexes to a thiol gradient, the peptides are
released from the DNA sequence according to their DNA
association constants. The strength of the disulfide
bond in a disulfide-linked peptide-DNA complex is direct-
ly related to the strength of the peptide-DNA associa-
30 tion. This relationship permits screening of tight-
binding peptides from a mixture of peptides. It is
reasonable to expect that the peptide that remains
complexed to the DNA sequence under conditions using the
highest concentration of thiol binds tightest to the DNA.
35 This screening process can be repeated in subsequent

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cycles with a peptide which has one additional amino acid
residue designated Xaa, in each cycle. The identifica-
tion of each Xaa residue can be determined by conven-
tional methods, such as peptide sequencing or UV absorp-
5   tion. The order of the next residue of the peptide,
resulting in the tightest binding to the DNA sequence is
determined.

Thus, the method described herein is a rational
method for the design, selection and production of mole-
10 cules that bind in a site-specific manner, to desired DNA
sequences. Examples of binding molecules include oligo-
meric molecules in which units can be added or removed
(e.g., D-, L-, or DL-peptides, peptidomimetic compounds
or complex carbohydrates).

15   Molecules made by the methods of the invention can
be used to regulate a wide variety of biological process-
es which depend on the site specific interaction of one
molecule with another molecule. For example, processes
mediated by the binding of a peptide with a nucleic acid,
20 or of a peptide with a peptide. Binding molecules which
bind with a nucleic acid can be used to prevent gene
activation by blocking the access of an activating factor
to its sequence element, repress transcription by stabi-
lizing duplex DNA or interfering with the transcriptional
25 machinery, or carry out targeted DNA modification by
delivering a reagent to a specific sequence. Binding
molecules which bind to peptides can be used to mediate
or otherwise participate in, various processes such as
antibody-antigen interactions, enzyme substrate interac-
30 tions, hormone-receptor interactions, and lymphokine-
receptor interactions.

Because the methods of the invention are chemical
rather than biological, they can be used to select or
discover binding molecules which are not normally synthe-
35 sized by living organisms, such as peptides which include

D-amino acids or nonbiogenic polymers (e.g., polymers derived from polyethylene glycol or nonnatural carbohydrates).

Methods of the invention described herein can be
5 used to optimize a single or small number of modifications, such as a single or small number of positions in a polymer, at each cyclic step and thus avoid steps in which extremely large numbers of species are screened.

Other advantages and features will become apparent
10 from the following descriptions and from the claims.

Brief Description of the Drawings

Figure 1 is a schematic representation of the reaction between a thiol-tethered oligonucleotide and a mixture of -SH-containing peptides.

15 Figure 2 is a graph of a hypothetical reduction-elution profile.

Figure 3 shows the components of the CGN4 binding system, including the oligonucleotides GCN4-1 (SEQ ID NO:1); GCN4-2 (SEQ ID NO:2); GCN4-3 (SEQ ID NO:3); GCN4-4
20 (SEQ ID NO:4) and the GCN4-derived peptide, including the disulfide tether (SEQ ID NO:5). The clear boxed area indicates the location of the tethered disulfide.

Figure 4 shows the results of coupling the disulfide-linked GCN4 peptide (SEQ ID NO:5) with the GCN4
25 oligonucleotides (SEQ ID NOS:1-4) as analyzed by denaturating polyacrylamide gel electrophoresis. X indicates what appears to be peptide-DNA complexes of differing mobility.

Detailed Description of the Invention

30 The present invention relates to methods of designing and producing a member of a binding pair which specifically binds to its partner as well as to the products resulting from these methods. Such members are referred

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to herein as specific binding molecules. It particularly
relates to methods of designing and synthesizing mole-
cules which specifically bind a desired DNA sequence
(i.e., sequence-specific or site-specific DNA binding
5 molecules).

    Specific binding molecule (also referred to herein
as binding molecule), as used herein, refers to an enti-
ty, e.g., a molecule, or a portion of a molecule, which
binds to a target. Preferably, a specific binding mole-
10 cule is susceptible to a plurality of successive or
serial modifications, e.g., in the case of a polymeric
molecule, the addition of monomeric units to the polymer-
ic chain. Preferably, the binding affinity of a specific
binding molecule with the target can be evaluated before
15 and/or after successive modification of the specific
binding molecule. A specific binding molecule is capable
of reversible attachment to a target, preferably via a
tether.

    Test-binding molecule (or test-molecule), as used
20 herein, refers to a specific binding molecule, some or
all of the structure of which is evaluated for inclusion
in the final structure of a specific binding molecule.
For example, in determining the structure of a peptide,
the intermediate or candidate peptides screened for
25 binding affinity are referred to as test-binding pep-
tides.   The specific binding molecule, e.g., a final
full length peptide, which is the product of the entire
process, can be referred to as a final or finished spe-
cific binding molecule.

30   Target, as used herein, refers to an entity with
which a specific binding molecule binds. Methods of the
invention optimize binding affinity between a target and
a specific binding molecule. A target can be a molecule,
a portion of a molecule, or an aggregate of molecules. A
35 target and a specific binding molecule can be separate

molecules, or they may be different moieties on one molecule. A target includes a target site. A target is capable of reversible attachment to a binding molecule via a tether. Examples of targets include: nucleic
5 acids (e.g., RNA or DNA, double stranded DNA, single stranded DNA, or supercoiled DNA), peptides or proteins (e.g., enzymes, receptors or antibodies), carbohydrates, and other molecular structures, such as nucleic acid-protein complexes, chromatin or ribosomes, lipid-bilayer
10 containing structures, such as membranes, or structures derived from membranes, such as vesicles.

Target site or specific site, as used herein, refers to a site on a target to which a specific binding mole-cule binds. Methods of the invention optimize binding
15 affinity between a specific binding molecule and a target site on a target. In the case of polymeric target mole-cules, a target site will usually include a specific sequence of monomeric subunits or a three dimensional structure. The actual structure (e.g., the chemical
20 structure, or three dimensional structure) of the target site need only be known with enough particularity to allow formation of a reversible bond to the target. Preferably, the molecular interactions between a binding molecule and a target site are noncovalent and have
25 energies of less than 25 kcal/mol at 25°C. These molecu-lar interactions include hydrogen bonds, Van de Waals interactions and electrostatic interactions.

Aggregate of molecules, as used herein, refers to two or more molecules which are connected by covalent or
30 noncovalent interactions.

Tether, as used herein, refers to a structure which includes a moiety capable of forming a reversible bond with another moiety (e.g., a moiety on another tether) and (optionally) a spacer element. Alkane chains are
35 suitable spacer moieties.

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Reversible bond, as used herein, refers to a bond
linking a binding molecule and a target (i.e., a binding
pair) which is thermodynamically stable but capable of
being broken by a reversing agent which is a physical or
5 chemical agent capable of breaking the bond. For any
given bond an appropriate reversing agent can be readily
chosen based on the chemical nature of the bond. For
example, a reversing agent for a disulfide bond is a
reducing agent such as thiol. The reversible bond is
10 between a tether on a specific binding molecule and a
tether on a target, a bond between tether on a specific
binding molecule and a target, a bond between a specific
binding molecule and a tether on a target, or a bond
directly between a target and a specific binding mole-
15 cule. By thermodynamically stable is meant a bond whose
strength is greater than 10, preferably greater than 20,
more preferably greater than 50, even more preferable
greater than 65, but preferably less than 100 Kcal/mol at
25°C.
20   Suitable examples of reversible bonds include: R_1-
$\underline{S-S}$-R_2, R_1-$\underline{S-Cd-S}$-R_2, and R_1-$\underline{S-Hg-S}$-R_2 wherein R_1 includes
a binding molecule or entity and R_2 includes a target and
the reversible bond is within the underlined area. Also
included are bonds in which a metal (e.g., Fe^{3+}, Co^{2+},
25 Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, or Hg^{2+}) is complexed between a
multidentate ligand (i.e., a ligand having two (or more)
moieties with which to complex an atom or group, prefera-
bly a metal atom) on a binding molecule, wherein a moiety
on the binding molecule can be, e.g., S, N, or an imidaz-
30 ole group, and e.g., a multidentate ligand on a target,
wherein a moiety on the target can be S, N, or an imidaz-
ole group. Examples of multidentate ligands follow:

5

10

$$R\text{---}N\begin{array}{c} \diagup \text{CH}_2\text{---}\text{CH}_2\text{---SH} \\ \diagdown \text{CH}_2\text{---}\text{CH}_2\text{---SH} \end{array}$$

15

20

$$R\text{---}N\begin{array}{c} \diagup \text{CH}_2\text{---}\text{CO}_2\text{H} \\ \diagdown \text{CH}_2\text{---}\text{CO}_2\text{H} \end{array}$$

25

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R
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5.

10

N   N
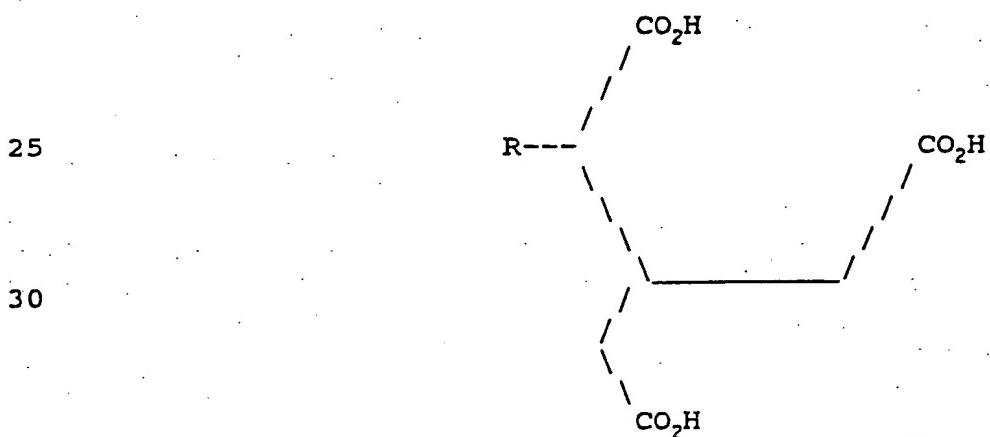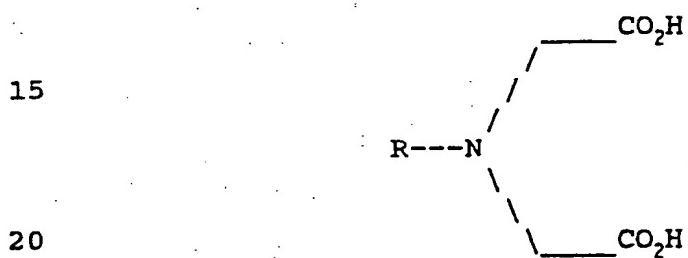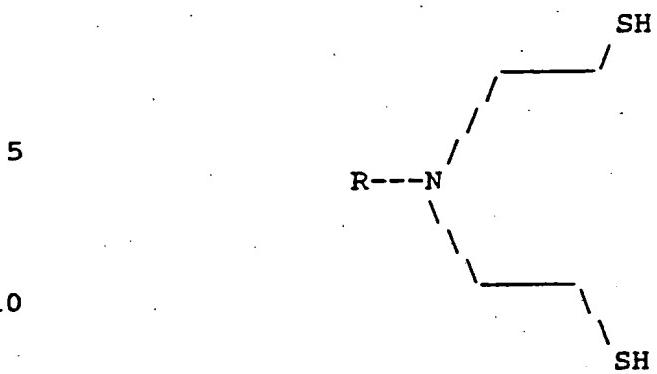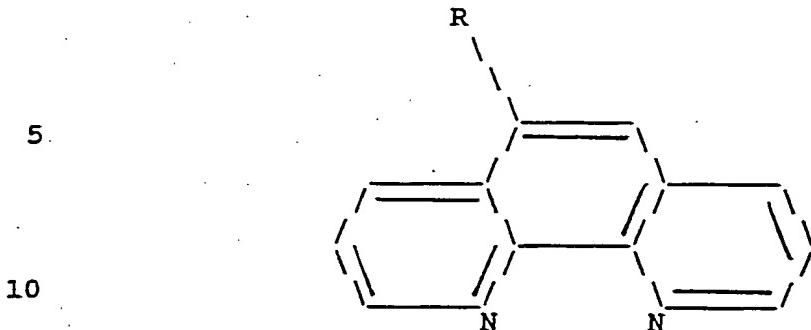
R----His$_6$

R---His-Gly-Gly

wherein R can be either a binding molecule or a target.
15 Any combination of multidentate ligands and monodentate
    ligands (i.e., a ligand having one moiety with which to
    complex a metal or other atom or group) can be used in
    the invention. For example, a binding molecule having a
    multidentate ligand and a target having a multidentate
20 ligand, a binding molecule having a monodentate ligand
    and a target having a monodentate ligand, or a binding
    molecule having a monodentate ligand and a target having
    a multidentate ligand can be used.
         Methods of the invention can be used to design
25 specific binding molecules which bind to a target site
    (i.e., a specific sequence) on a target molecule. These
    methods include an iterative process comprising succes-
    sive cycles of: (1) modifying a test-binding molecule
    (also referred to as a test-molecule); and (2) evaluating
30 the affinity of the modified test-binding molecule for a
    target site on the target molecule. The evaluation

includes evaluating the relative affinity of a test-
binding molecule for a target site as compared with other
test-binding molecules in a pool, or mixture of test-
binding molecules. The affinity of the test-binding
5 molecule for the target can be determined by forming a
reversible bond between the test-binding molecule and the
target. The susceptibility of the reversible bond to
reversal is related to the affinity of the test-binding
molecule for the target site on the target. In most
10 applications a number of species of test-binding mole-
cules, representing alternative modifications of a test-
binding molecule (i.e., modifications of the initial
test-binding molecule or a test-binding molecule from the
previous cycle of the method) are evaluated simultaneous-
15 ly at each cycle. The structure of the species (at each
cycle) which gives the optimum results is chosen to
supply an element of the structure of the final specific
binding molecule.

Thus, application of the method described herein,
20 results in the elucidation of a preferred structure for
the final binding molecule. While any molecule or combi-
nation of molecules which can be subjected to such a
process can be used as a test-binding molecule, a partic-
ularly useful application of methods described herein,
25 involve the generation of DNA binding peptides.

The synthesis and identification of a peptide which
can bind to a sequence specific target site on a target
DNA molecule can be performed as follows. A moiety
capable of forming a reversible bond with a moiety on the
30 test-binding molecule is attached to target DNA mole-
cules. For example, a sulfhydryl group is tethered by an
alkane chain to a site such as a site in a major or minor
groove in a DNA molecule. In one embodiment, the DNA-
$[C]_n$-SH is then attached to an immobilizing matrix. The
35 DNA-$[C]_n$-SH molecules are then complexed, via a disulfide

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bond, to a mixture of synthetic peptides and placed in a
chromatography column as shown in Figure 1. X in Figure
1 represents the number of species of peptides in a
mixture of peptides. The curved line connecting the
5   peptide to the DNA target represents the tether. The
vertical arrows between the peptide and the DNA target
represent the specific binding molecule/target site
interaction, which, preferably, is the interaction the
method optimizes.

10   The synthetic peptides are all of the formula CO_2H-
Cys-Xaa-NH_2 (where Xaa equals any amino acid residue
which lacks an -SH group). Either or both the N or C
terminal can be modified, or blocked, as in the structure
HN_2CO_2-Cys-Xaa-$NHCO_2CH_3$, to prevent unwanted interaction
15   between the specific binding molecule and the target.
Amino acids may be added at either end of the molecule.

        The mixture of synthetic peptides includes a variety
of species (i.e., a plurality of peptides of different
sequences) with differences in sequences arising from
20   various candidate residues occupying the second (Xaa)
position in different peptides. The candidate residues
may be any moiety which lacks an -SH group and which can
be incorporated into the peptide chain, including, for
example, D- or L-amino acids, naturally occurring or non-
25   naturally occurring amino acids, or α-, β-, or γ- amino
acids.

        The test-binding molecule will have different bind-
ing affinities for the target DNA sequence, and these
differences will affect the reducibility of the disulfide
30   bond between the peptide and the DNA molecule with which
it is complexed. In one embodiment, passage of a thiol
gradient through the peptide-DNA column results in the
release of the peptides according to the susceptibility
of the binding molecule-target disulfide bond to reduc-
35   tion (i.e., reversal). This results in an elution pro-

file which reflects the differences in susceptibility to reduction and thus the differences in the target DNA binding constants between the various dipeptides and the target. The later a dipeptide elutes, the higher its

5 binding affinity for the target DNA sequence. Inspection of the elution profile of the dipeptides allows determination of the optimal residue at the second position. Figure 2 shows a hypothetical elution profile. The concentration of thiol is represented by a dashed line

10 and the elution profile by a solid line. The peak labeled A represents the species with the highest binding affinity for the target.

The entire process is repeated with a set of tripeptides. For example, CO_2H-Cys-XAA-Xaa-NH_2, where XAA is

15 the optimum second position residue and Xaa is defined as above, is cycled through the process to determine the optimum residue for the third position in the binding peptide. Subsequent cycles extend the sequence of the binding peptide to the desired length. The desired

20 length can be a predetermined number of amino acid residues, or can be a length at which the binding molecule exhibits useful or optimum binding affinity and/or sequence specificity.

While the peptides are lengthened by one residue per

25 cycle in the above example, it is also possible to perform more than one modification, (e.g., to add 1, 2, 3, 4, or more residues) per cycle. When used in conjunction with conventional solid-phase-peptide synthesis technology, this strategy allows the generation of DNA binding

30 peptides of desired lengths.

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Choice of the Reversible Bond or Tether Sites

     The site at which the reversible bond or tether is
placed (on both specific binding molecule and target)
should be chosen so as to allow a specific binding
5 molecule coupled to the target unhindered access to the
target site on the target. Stearic hindrance imposed by
the location or structure of the bond or tether(s) can
interfere with the correlation between bond reversibility
and binding molecule-target site affinity. The inclusion
10 of a spacer element can reduce stearic hindrance. For
example, an alkane of appropriate length can be used to
provide both flexibility and sufficient separation be-
tween the binding molecule and the target site.

     When a nucleic acid is the target molecule a nucleic
15 acid of any strandedness and of any topology can be used
in methods of the invention. In the case of double
stranded DNA, the tether can be located in a major or
minor groove close to the target sequence, but not so
close as to result in stearic hindrance to binding from
20 strain on the bond between the binding peptide and the
target.

     The reversible bond or tether can be located such
that either binding molecule-target interactions or
binding molecule-solution interactions are favored. For
25 example, in the case of an essentially linear target,
such as double stranded DNA, the reversible bond or
tether can be placed at or near a terminus of the mole-
cule to favor binding molecule-solution interactions, or
in the central areas (away from the termini), to favor
30 binding molecule-target interactions.

     A tether can be attached to DNA, or the reversible
bond formed, on a base at any exocyclic amine or any
vinyl carbon, such as the 5 or 6 position of pyrimidines,
8 or 2 positions of purines, at the ultimate 5' or 3'

carbons, at the sugar phosphate backbone, or at
internucleotide phosphorus atoms.

Choice of Reversible Bonds and Tethers

In methods of the invention described herein, the
5 binding molecule is conjugated to, or associated with,
the target by a reversible bond. In some embodiments the
reversible bond is between a tether on the target and a
tether on the specific binding molecule. In embodiments
with two tethers, the tether on the binding molecule can
10 be the same as the tether used on the target. Alterna-
tively, different tethers can be used on each. In other
embodiments only one tether is used, and in some embodi-
ments the reversible bond is formed directly between the
binding molecule and the target.
15 The tethers and the reversible bond should have the
following characteristics. A tether (or reversible bond)
should be capable of attachment to the target without
substantial alteration of the three dimensional structure
of the target. For example, the reversible bond or
20 tether-bearing-target should remain similar enough in
conformation to the _in vivo_ target so that the binding
molecules generated will recognize and bind to the _in
vivo_ target with a useful affinity and site specificity.
Additionally, the reversible bond formed between the
25 target and the binding molecule should reversibly couple,
by a covalent or ionic bond, the target to the binding
molecule. The susceptibility to reversal, or breakage,
of the reversible bond formed between the target and the
binding molecule should vary with the affinity of the
30 binding molecule for the target site on the target. The
tether or tethers should be of appropriate length and
flexibility such that the binding molecule has free
access to the target site, and under the conditions used
in methods of the invention, the reversible bond and/or

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tethers should be substantially unreactive with other
sites on the binding molecule or target molecule.

     Thiol groups are suitable moieties for forming a
reversible bond.   A reversible bond, e.g., a disulfide or
5 metal-bridged disulfide bond, formed between -SH groups
can be broken by contacting the bond with a reducing
agent.   In the case of a metal bridged disulfide, the
reversible bond can be reversed with a ligand which
competes with the metal atom for its position in the
10 bridge.   When the binding molecule is a peptide, the
amino acid residue, cysteine, is a convenient source of
an -SH group for use as the binding molecule tether.
Alkane chains are suitable spacer moieties.

     Methods for attaching tethers to targets, such as
15 nucleic acid molecules, are known to those skilled in the
art.   (MacMillan et al., Tetrahedron 47:2603-2616 (1991);
MacMillan et al., J. Org. Chem. 55:5931-5933 (1990);
Ferentz et al., J. Am. Chem. Soc. 113:4000-4002 (1991);
Zuckerman et al., Nuc. Acid Res. 15:5305 (1987); Connolly
20 et al., Nuc. Acid Res. 13:4485 (1985); Letsinger et al.,
J. Am. Chem. Soc. 103:7394-7396 (1981); Fidanza et al.,
J. Am. Chem. Soc. 111:9117-9119 (1989)).

     In one embodiment of the method described herein,
where the reversible bond between the binding molecule
25 and the target is disrupted with a reversing agent, it is
convenient to immobilize the target molecule before
exposure to the reversing agent.   This can be done by
attaching, or linking the target to a matrix, such as a
resin.   Methods for attaching molecules to resins are
30 known to those skilled in the art.

Formation of Test Binding Molecule-Target Complexes

     Test-binding molecules (i.e., putative or candidate
binding molecules) can be synthesized by methods known to
those skilled in the art.   As described in the Example, a

derivative of the DNA binding protein, GCN4, (O'Shea, E. K., et al., Science 243:538-542 (1989); Talanian, R. V., et al., Science 249:769-771 (August 1990); Talanian, R. V., et al., Biochem. 31:6871-6875 (1992)) was synthe-

5 sized. The GCN4-derived peptide is a monomer, comprised of 24 amino acid residues (SEQ ID NO:5).

Also as described in the Example, four modified DNA oligonucleotides, carrying a tethered disulfide at four different positions with respect to the CGN4-binding site

10 (Figure 3, SEQ ID NOS:1-4) were synthesized using known methods. (MacMillan, A. M., and Verdine, G. L., J. Org. Chem. 55:5931 (1990); Ferentz, A. E., and Verdine, G. L., J. Am. Chem. Soc. 113:4000-4002 (1991).

The peptide was reduced, also as described in the

15 Example, and, using the reaction conditions described in the Example, formation of the disulfide bond between the CGN4-derived peptide and the four DNA oligonucleotides was carried out. After incubation of the coupling reaction mixture, aliquots were taken and analyzed on poly-

20 acrylamide gels under denaturing or native conditions.

Figure 3 shows the results of the analysis of aliquots from the four reaction mixtures containing the CGN4-derived peptide and the modified DNA sequences, on a denaturing gel. In all four reaction mixtures, a disul-

25 fide-linked GCN4 peptide-DNA complex was formed, as indicated by the arrows denoting uncomplexed DNA and peptide-DNA complexes.

The structures of the disulfide-linked GCN4-DNA complexes were also analyzed to determine whether the

30 peptides associated with the DNA oligonucleotides in a way that mimics their natural counterparts, or at least to discern that the peptide is bound in a sequence-spe- cific manner. Preliminary data using DNA footprinting techniques (Galas, D. J. and Schmitz, A., Nucleic Acid

35 Res. 5:3157-3170 (1978) indicate that three out of the

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four modified DNA oligonucleotides bound the GCN4-derived
peptide in the anticipated region. That is, the data is
strongly suggestive that the peptide bound to three DNA
sequences in a site-specific manner.

5   In one embodiment, binding of peptides to thiol-
tethered DNA via formation of a disulfide bond can be
performed as follows. Peptides can be bound quantita-
tively to a thiol-tethered DNA molecule that is bound to
a polymer resin, by formation of a disulfide bond between
10 the DNA and the peptides. In these experiments, the
object is to bind approximately 100% of the peptides to
the resin-bound DNA, hence, an excess (2-10-fold mole
excess based on the thiol-containing DNA strand) of
resin-bound DNA, relative to moles of thiol groups (or
15 disulfide groups) on the peptides is used.

The resin-bound DNA is prepared in the reduced state
by treatment with common disulfide-reducing agents
(alkanethiols or borohydride compounds). This incubation
can be done in a batch mode or by passage of reagents
20 through a column containing the resin-bound DNA. The
excess reducing agents can be removed by filtration
(batch mode) or elution (column mode).

Charging of the peptides onto the resin can either
be done in batch mode or column mode. In either case,
25 the thiol group of the peptides will first be activated
by conversion to the corresponding 2-thiopyridyl or 5-
thio-2-nitrobenzoyl disulfide, using standard methods.
The activated peptides, in deaerated buffer, pH 7-9 (for
example 50 mM Tris, pH 8.0) will be incubated with the
30 reduced DNA-bound resin either with shaking or stirring
(batch mode) or with recirculation (column mode). Alter-
natively, the resin-bound DNA can be prepared as the 2-
thiopyridyl or 5-thio-2-nitrobenzoyl disulfide, and the
reduced peptides bound as described above.

The binding reactions can be quantified by UV measurements, monitoring release of the pyridine-2-thione or 5-thio-2-nitrobenzoate chromophores. Alternatively, the amount of peptides bound to the resin or free in solution
5 can be quantified by a routine ninhydrin test. The presence of free thiol groups on any material at any stage of the experiments can be monitored by alkylation with ^{14}C-iodoacetamide.

Binding can be optimized by examination of % pep-
10 tides bound versus method of activation (DNA-disulfide or peptide-disulfide), activating agent (2-thiopyridyl or 5-thio-2-nitrobenzoyl), binding mode (batch or column), time of incubation, temperature, and structure of the thiol-containing tether in the DNA.

15 In another embodiment, equilibrium binding of peptides to thiol-tethered DNA via formation of a disulfide bond can be performed. Peptides can be bound under equilibrium conditions to a thiol-tethered DNA molecule that is bound to a polymer resin, by formation of a
20 disulfide bond between the DNA and the peptides. The disulfide bond between the DNA and peptides can be formed under freely reversible conditions, so the noncovalent interaction of the peptide with DNA will cooperate with the covalent interaction (i.e., disulfide bond formation)
25 to establish a stable complex. These experiments can be carried out in a batch mode.

The thiol-tethered DNA is mixed with a stoichiometric amount of the peptides in a deaerated redox buffer. The redox buffer can be the same as the redox eluent
30 described above. The most important components are the reduced and oxidized forms of a thiol reducing agent, such as 2-thiopyridine, 5-thio-2-nitrobenzoate, dithiothreitol, 2-mercaptoethanol, and N,N'-dimethyl-N,N'-bis(mercaptoacetyl)hydrazine (DMH). The reactants
35 are allowed sufficient time to reach equilibrium. Alter-

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natively, if the DNA is resin-bound, then the resin is
pelleted by centrifugation, and the supernatant is re-
moved. The pellet is washed with buffer (lacking added
thiols or disulfides) and pelleted again. DNA-bound
5 peptides are then eluted by incubation of the resin under
strongly reducing conditions (such as 100 mM dithiothre-
itol). Ordinarily, parallel incubations (containing
different relative amounts of the reduced and oxidized
forms of the thiol reducing agent) should be set up and
10 analyzed separately.

     The following conditions can be varied to optimize
the system: chemical structure of redox eluent, concen-
tration of redox eluent, temperature, flow rate, buffer
conditions (pH, ionic strength, addition of organic co-
15 solvents such as trifluoroethanol).

     Peptides can be quantified by amino acid analysis
and sequenced by automated phenylthiohydantoin methods.

Determination of Binding Molecule-Target Site Binding
Affinity

20   The affinity of a specific binding molecule for the
target site on a target can be determined by evaluating
the ease with which a reversible bond between the binding
molecule and the target can be reversed. These determi-
nations can be made by immobilizing the binding molecule-
25 target complex, such as on a matrix or a resin, and
passing a gradient of a reversing agent (an agent which
reverses, that is, breaks, or disrupts, the reversible
bond and thus releases the binding molecule from the tar-
get site) over the immobilized complexes.

30   In most embodiments of the methods described herein,
several species (also refrred to herein as a plurality)
of test-binding molecules will be screened simultaneously
to determine which test-molecule possesses the optimum
binding properties. The elution profile allows determi-

nation and comparison of the binding affinities of various species of test-binding molecule and selection of the species which represents the optimum or desired structure for the final specific binding molecule.

5   In the case of a peptide binding molecule complexed to a DNA target molecule by a disulfide bond, the resin bound peptide-DNA complexes are placed in a chromatography column. A gradient of a reducing agent, e.g., a thiol reagent, is applied to the column. This results in

10   the release of peptides according to their DNA association constants, producing a reductive elution profile. The peptide that elutes last has the highest affinity for the target DNA. This chemical screening process thus provides the optimal residue at the tested position.

15   Elution of peptides coupled to a target by a disulfide bond can be performed, either in batch or column mode, as follows. Column mode allows more precise control over the elution conditions, since the column can be attached to a commercially available gradient elution

20   system, such as the Fast Protein Liquid Chromatograph (FPLC), Pharmacia) or any similar apparatus. Batch mode operation may be necessary if the conditions required for elution (e.g., high temperatures, long elution times) are incompatible or inconvenient with FPLC.

25   In the column mode, a redox gradient is passed through the column, causing peptides to be released depending on their redox potential. In the simplest case, the redox gradient consists of mixtures of a thiol or dithiol compound and its corresponding disulfide. In

30   the beginning of the gradient, the redox eluent contains 100% of the disulfide form, and at the end of the gradient, 100% of the thiol (or dithiol) form. Typical redox eluents consist of the thiol and disulfide forms of 2-thiopyridine, 5-thio-2-nitrobenzoate, dithiothreitol, 2-

35   mercaptoethanol, and the N,N'-dimethyl-N,N'-bis(mercapto-

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acetyl)hydrazine (DMH) reagent recently reported by
Whitesides (J. Org. Chem. 56:2332-2337 (1991)). The
latter may be preferable because of its exceptionally
fast kinetics of disulfide reduction.

5   Elution of peptides from the column is monitored by
on-line UV detection at 214 nm and post-column derivati-
zation with ninhydrin. Peptides are quantified by amino
acid analysis and sequenced by automated phenylthiohydan-
toin methods.

10   The following conditions can be varied to optimize
elution for speed, ease, or resolution: chemical struc-
ture of redox eluent, concentration of redox eluent,
slope of gradient, shape of gradient (linear, step,
exponential), temperature, flow rate, buffer conditions
15 (pH, ionic strength, addition of organic co-solvents such
as trifluoroethanol).

       In the batch mode, the resin containing DNA-bound
peptides is incubated in an Eppendorf tube with deoxygen-
ated buffer containing the redox eluent. Redox eluents,
20 quantification and identification of peptides are the
same as described above for the column mode. The follow-
ing conditions can be varied to optimize elution: chemi-
cal structure of redox eluent, concentration of redox
eluent, number and spacing of stepwise elutions, elution
25 time, temperature, buffer conditions (pH, ionic strength,
addition of organic co-solvents such as trifluoroetha-
nol).

       After the determination of a first optimum modifica-
tion (i.e., the determination of the optimum residue at a
30 given position of a specific binding molecule) has been
made, a second modification can be performed on the test-
binding molecule (e.g., the addition of a subsequent
residue to a polymeric binding molecule) and the process
of evaluating the binding affinity of the newly modified

test-binding molecule repeated. This cycle may be re-
peated a number of times.

As in the first cycle, it will usually be desirable
to simultaneously evaluate a number of species (i.e., a
5 plurality) of test-binding molecules (representing a
number of different modifications) at each cycle or
iteration. For example, in the case of a peptide binding
molecule, a plurality of peptide species, differing by
the residue at the position (or positions) being opti-
10 mized, are tested simultaneously. The structure (e.g.,
in the case of a peptide binding molecule, the particular
residue) giving optimum results is selected.

In the case of a peptide binding molecule, a DNA
target molecule, and -SH tethers, the following protocol
15 can be used. After the optimum amino acid residue at the
second position is determined, a set of tripeptides of
the formula CO_2H-Cys-XAA-Xaa-NH_2 (where XAA is the optimum
second position amino acid and Xaa represents any amino
acid which lacks an -SH group), is synthesized. Each
20 peptide of the set differs at Xaa. The elution and
determination of binding affinity is repeated with the
tripeptide to yield the optimum amino acid residue at the
third position. The process is repeated until the de-
sired length is reached.

25 After the iterative methods of synthesis and selec-
tion described above have been used to generate the
sequence order and structure of a binding molecule,
further modifications can be performed on the binding
molecule. These modifications may be in the form of a
30 second round of selected optimizations of a different
binding molecule characteristic. For example, after an
initial determination of the optimum primary sequence of
a peptide, a second iterative selection can be applied to
determine an optimum level of glycosylation, the effect
35 of cofactors, the effect of homo- or heterodimerization,

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or the effect of inter- or intra-chain cross linking.
These, or other modifications may be tested for their
effect on binding by non-iterative methods as well.
Additionally, a second iterative selection can be per-
5 formed to select a second specific binding molecule to
form a heterodimer with the binding molecule selected in
the first iterative cycle. These two specific binding
molecules may be cross-linked by conventional methods.
      Modifications such as the formation of homo- or
10 heterodimers, may require alteration of a selected bind-
ing molecule. For example, new peptides may be
constructed to optimize the spacing of binding units
relative to each other and the center of target sites in
the DNA, or to allow the introduction of specifically
15 desired residues. Molecular modeling can be used to
facilitate the choice of modifications. The sequence
specificity of dimerized peptides can be tested by meth-
ods known to those skilled in the art (e.g., by competi-
tion electrophoretic mobility shift assays, PCR-based
20 target detection assay, or chemical or enzymatic
footprinting).

Optimization of Conditions for Determining Binding
Affinity
      General conditions under which the reversible bond
25 between the binding molecule and the target are formed
and broken, and the methods of evaluation of the rela-
tionship between reversible bond breakage and binding
molecule/target site binding affinity, can be determined
by practicing the methods described above with relatively
30 well characterized molecules, as is exemplified in the
Example with the GCN4 system.
      In addition to the GCN4 system, the X-ray crystal
structures of the bacteriophage repressor (Jordan et al.,
Science 242:893 (1988)) and the murine Zif268 protein

(Pavletich et al., Science 252:809 (1991)) bound to their
respective DNA sites are deposited in the Brookhaven
Protein Data Bank. These can also be retrieved and
molecular modeling methods used to trim the structures
5 down to a peptide-bound DNA core structure, as was done
with GCN4. Disulfide tethers can be designed to link the
resulting peptides to DNA, bearing in mind that the
connector should be as short as possible without generat-
ing strain. The λ repressor and Zif268 systems are
10 favorable for optimization because they represent respec-
tively, examples of extended and α-helical peptides that
bind DNA as isolated units and for which high-resolution
structures in the DNA-bound form are available. The α-
helices of Zif268, while being part of a zinc finger
15 structural motif, possess all of the residues of that
motif that are involved in base-contacts.
     DNA-binding peptides designed on the basis of X-ray
structures (hereafter referred to as "wild-type" pep-
tides) can be synthesized by standard methodology.
20 Thiol-tethered oligonucleotides designed similarly
("wild-type" oligonucleotides) can be synthesized by
methods and linked to a resin, as described above.
The peptides can be tethered to DNA both in solution (for
use in high-resolution structural studies) and on a solid
25 matrix (for reductive elution studies). The conditions
for forming and releasing the peptide-DNA reversible bond
can be optimized using these molecules, as described in
the Example. Systems having sequence changes in the DNA
or peptide ("mutant" oligonucleotides or peptides) that
30 should disrupt sequence-specific peptide-DNA interac-
tions, can be synthesized in parallel for use as controls
or to further investigate elution conditions.
     The structures of the DNA-tethered peptide systems
constructed in the previous state can be evaluated to
35 discern whether the peptides are associated with DNA in a

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way that mimics their natural counterparts, or at least
in a way that is discernibly sequence-specific. ^{1}H-NMR,
^{15}N-NMR, chemical footprinting, and circular dichroism
spectroscopy can be used to evaluate these molecules.

5   Wild-type and mutant peptide-DNA systems, assembled
on a solid matrix in a column can be subjected to reduc-
tive elution by a thiol gradient. Parameters affecting
elution, such as reducing agent, temperature, pH and
slope of the gradient, can be optimized. For example,
10 this approach can be used to find conditions in which
wild-type λ and Zif268 peptides are strongly retained
(elute late in the gradient) while peptide from mutant
systems are not strongly retained (elute early).

       Following optimization of the reductive elution
15 conditions for the elongation of wild-type peptides,
screening of peptide mixtures can be optimized. The
wild-type peptides can be elongated by one peptide unit,
using a mixture of any amino acids that lack an -SH
group. This 19 peptide mixture can then be coupled to
20 the solid matrix, loaded into a column, and eluted
reductively. The late-eluting peptides will be sequenced
(e.g., by fast atom bombardment mass spectrometry and/or
phenylthiohydantoin degradation). This synthesis and
screening process can be repeated iteratively until
25 either the efficiency of synthesis or resolution of the
column procedure falls off.

       Elongated peptides that are obtained by iterative
selection should bind selectively to longer target DNA
sequences than the starting peptides. The interaction of
30 these peptides with DNA can be studied by the same meth-
ods as described above for the starting peptides.

       Moreover, the three dimensional molecule can serve
as a guide in choosing the modifications. This can allow
the optimization of residues on the same face or side of
35 a structure. For example, in the case of a binding mole-

cule which is a helical molecule, it may be desirable to
add subunits in groups of n, where n is the number of
subunits involved in one full turn of the helix. In the
case of an α-helical protein, wherein n=3.6 residues

5 could be added in groups of 3, with the first two of the
three being held constant (e.g., the first two residues
being predetermined residues) or in groups of 4 with the
first three of the four being held constant (e.g., con-
sisting of predetermined residues) with the final resi-

10 due, in either case, being varied.

An analogous method can be used to optimize the
residues on one face of a β-sheet or β-ribbon structure.
Since residues i, i + 2, i + 4, i + x, will be on the
same surface of a β-ribbon or a β-sheet structure, resi-

15 dues can be added as tripeptide, with the final residue
of the peptide being varied.

The desired three-dimensional structure of the
binding molecule can also influence choice of modifica-
tion in other ways. For example, in the case of a pep-

20 tide, residues which promote the formation of a helical
structure, such as 2-aminoisobutyric acid or α-methyl
amino acids, can be added. Similarly, pro-gly could be
added to a sequence to interrupt a helical structure. A
pro-gly series can be added to a peptide sequence to

25 introduce a fold in a β-sheet or β-ribbon structure.

Peptide-on-phage libraries can be used to supply the
binding entities in methods of the invention. For exam-
ple, a fully degenerate phage library could include all
peptide test-binding entities to be tested in one batch.

30 The peptides could be coupled to the target and eluted as
a batch.

The invention will now be illustrated further and
more specifically by the following Exemplification.

<u>Example: Formation of Disulfide-linked-peptide-DNA Complexes</u>

1. Synthesis and purification of peptides

All GCN4-derived peptides were synthesized on Ap-
5 plied Biosystems Model 431A peptide synthesizer with
standard reaction cycles. Peptides were deprotected and
cleaved from the resin by incubation in the mixture of
trifluoroacetic acid:phenol:anisole:ethanedithiol
(94:2:2:2) for 4 hours at room temperature. The peptide
10 solution was precipitated and washed 4-5 times with ice-
cold diethyl ether. The pellet was dried with air,
dissolved in 1ml of 10% acetic acid and lyophilized. The
peptide was purified by HPLC with ZORBAX reverse-phase C-
8 semi-preparative column (DuPont Instruments) and a
15 linear gradient of acetonitrile-water with 0.1% TFA.
Fast atom bombardment mass spectroscopy revealed a peak
at 2613.07 which agrees with the calculated mass of
2611.97. Collected fractions were lyophilized and stored
at -20°C.

20   ### 2. Synthesis and purification of DNA oligonucleo-
tides

All oligonucleotides were synthesized on an Applied
Biosystems DNA synthesizer Model 381A using conventional
and modified phosphoramidites according to the "convert-
25 ible nucleoside approach" described in MacMillan, A. M.
and Verdine, G. L., <u>J. Org. Chem.</u> <u>55</u>:5931 (1990) and
Ferentz, A. E., and Verdine, G. L., <u>J. Am. Chem. Soc.</u>
<u>113</u>:4000-4002 (1991). The displacement reaction was done
with the disulfide of aminepropanethiol to yield modified
30 oligonucleotides with N^6-thioalkyl-dA or N^4-thioalkyl-dC,
protected as mixed disulfides. Both modified and unmodi-
fied oligonucleotides were purified by polyacrylamide gel
electrophoresis (PAGE) on 20% denaturing gels.

Annealing of different modified oligonucleotides
with the corresponding complementary strands produced
four double-stranded probes carrying the tethered disul-
fide at four different positions with respect to the
GCN4-binding half-site. (Figure 2; GCN4-binding half-
site shaded in gray).

3. Reduction of peptides

The lyophilized GCN4-derived peptide was dissolved
in 0.1 ml of 1xTE8 (Tris-EDTA buffer, pH 8) and peptide
concentration determined by UV spectroscopy (210 and 220
nm) was 3 mM. The peptide was reduced by the addition of
1 microliter of 1:10 dilution of 2-mercaptoethanol stock
(14.4M, obtained from Bio-Rad Laboratories) and incubated
at 50° for 30 minutes. The reaction mixture was subse-
quently lyophilized in the speedvac concentrator (Savant)
to evaporate 2-mercaptoethanol and the dry pellet was
dissolved in 0.1 ml of 10xTE8.

4. Coupling reaction and the analysis of results

The disulfide bond between the peptide and DNA was
formed by mixing the 5-10 pmols (20-80K CPM) of the [32]P
end-labeled double stranded DNA probe with different
amounts (5pmols-5nmols) of reduced GCN4-derived peptide
in the buffer containing 50 mM KCl, 20mM Tris pH 7.5 and
10% glycerol. The coupling reaction mixture (20 micro-
liters) was incubated at room temperature for 8-48 hours.
Aliquots (2-4K CPM) from each reaction were analyzed on
denaturing (Figure 3) or native 20% acrylamide gels, and
by DNA footprinting.

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Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the
5 invention described herein. Such equivalents are intended to be encompassed by the following claims.

The invention claimed is:

CLAIMS

1. A method of designing and producing a specific binding molecule, comprising the steps of:

   a) combining: 1) a desired target containing a first moiety capable of forming a reversible bond with a second moiety and; 2) a test-molecule comprising a unit to be assessed for its ability to bind a region of the desired target and containing the second moiety, thereby producing a combination;

   b) maintaining the combination produced in (a) under conditions appropriate for formation of a reversible bond between the first moiety and the second moiety, and binding of the unit to be assessed with a region of the desired target and the test-molecule, thereby producing desired target - test-molecule complexes;

   c) subjecting complexes produced in (b) to conditions which result in reversal of the reversible bond, thereby producing a mixture which contains complexes, uncomplexed desired target molecules, and test-molecules;

   d) determining the identify and order of test-molecules present in the complexes; and

   e) repeating steps a) through d) in a series of cycles, wherein in each subsequent cycle, test-molecules in step (a) comprise one unit more than in the preceding cycle and the test-

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molecules in complexes formed in step
(b) comprise one unit more than test-
molecules present in complexes formed
in step (b) of the preceding cycle.

5   2.   A specific binding molecule produced by the method
           of Claim 1.

     3.   A method of designing and producing a sequence-
           specific DNA binding molecule, comprising the
           steps of:
10   a)   combining: 1) a desired DNA sequence
                 containing a first moiety capable of
                 forming a reversible bond with a sec-
                 ond moiety and; 2) a test-molecule
                 comprising a unit to be assessed for
15   its ability to bind a region of the
                 desired DNA sequence and containing
                 the second moiety, thereby producing
                 a combination;
           b)   maintaining the combination produced
20   in (a) under conditions appropriate
                 for formation of a reversible bond
                 between the first moiety and the sec-
                 ond moiety, and binding of the unit
                 to be assessed with a region of the
25   desired DNA sequence and the test-
                 molecule, thereby producing desired
                 DNA sequence - test-molecule com-
                 plexes;
           c)   subjecting complexes produced in (b)
30   to conditions which result in rever-
                 sal of the reversible bond, thereby
                 producing a mixture which contains
                 complexes, uncomplexed target DNA

sequences, and uncomplexed test-mole-
cules;

d)   determining the identify and order of test-
molecules present in the complexes; and

5   e)   repeating steps a) through d) in a
series of cycles, wherein in each
subsequent cycle, test-molecules in
step (a) comprise one unit more than
in the preceding cycle and the text

10   molecule in complexes formed in step
(b) comprise one unit more than test-
molecules present in complexes formed
in step (b) of the preceding cycle.

4.   A sequence-specific DNA binding molecule produced by
15   the method of Claim 3.

5.   A method of Claim 2 wherein the test-molecule of
step a) is a peptide and the unit to be assessed is
an amino acid residue.

6.   A sequence-specific DNA binding molecule produced by
20   the method of Claim 5.

7.   A method of Claim 5 wherein the reversible bond of
step b) is a disulfide bond formed between an -SH
group on the test-molecule and an -SH group on the
desired DNA sequence.

25 8.   A sequence-specific DNA binding molecule produced by
the method of Claim 7.

9.   A method of Claim 3 wherein step c) further compris-
es subjecting complexes to a reversing agent.

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10.   A method of Claim 5 wherein the reversing agent is a
      reducing agent.

11.   A sequence-specific DNA binding molecule produced by
      the method of Claim 10.

5 12.   The method of Claim 3, wherein the desired DNA se-
         quence comprises a DNA molecule comprising an -SH
         group, the test-molecule comprises an -SH group, the
         reversible bond formed between the -SH groups is a
         disulfide bond and the reversing conditions comprise
10   subjecting complexes to a reducing agent to break
         the disulfide bond.

13.   A sequence-specific DNA binding molecule produced by
      the method of Claim 12.

14.   The method of Claim 12, further comprising attaching
15   the DNA molecule to an immobilizing matrix, and
         wherein subjecting complexes to the reducing agent
         comprises contacting the complex with a concentra-
         tion gradient of the reducing agent, and determining
         the ability of the reducing agent to disrupt the
20   disulfide bond comprises determining the ability of
         the reducing agent to elute the test-molecule from
         the immobilized DNA.

15.   The method of Claim 14, wherein the test-molecule
      comprises a peptide comprising a first and second
25   subunit, the first subunit comprises a first amino
         acid residue comprising an -SH group and the second
         subunit comprises a second amino acid residue which
         does not contain an -SH group.

16.   The method of Claim 15, wherein the first subunit comprises cysteine.

17.   The method of Claim 12, wherein

step a) further comprises providing a plurality of test-molecules comprising a plurality of sequences, each of the test molecules comprising a first subunit comprising an -SH group and a second subunit which does not contain an -SH group,

step b) further comprises maintaining a plurality of the test-molecules with a plurality of the DNA molecules to form a plurality of complexes, each of the complexes comprising a test-molecule linked by a disulfide bond to a DNA molecule,

step c) further comprises subjecting a plurality of the complexes to a reducing agent to break the disulfide bonds; and

step d) further comprises determining the susceptibility of the bonds to the reducing agent as an inverse measure of the ability of a test-molecule to bind to the DNA molecule, the sequence of the test-molecule comprising the sequence of the test-molecule of the complex with the disulfide bond most resistant to breakage by the reducing agent.

18.   The method of Claim 3, wherein the test-molecule is of a predetermined length and the method further comprises comparing the length of the sequence generated in step (d) with the predetermined length and if the desired length has not been reached, then adding another subunit to the subsequent test-molecule and repeating steps (a) through (d).
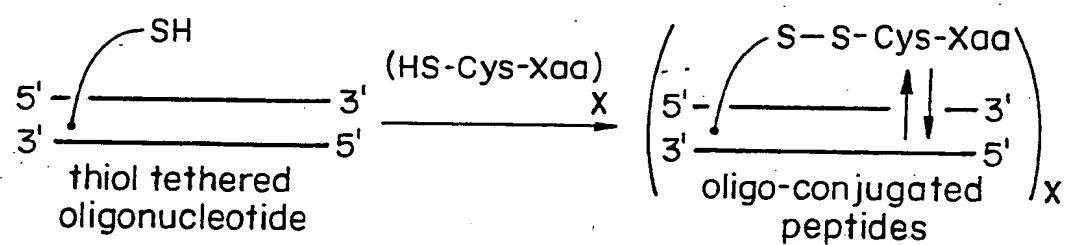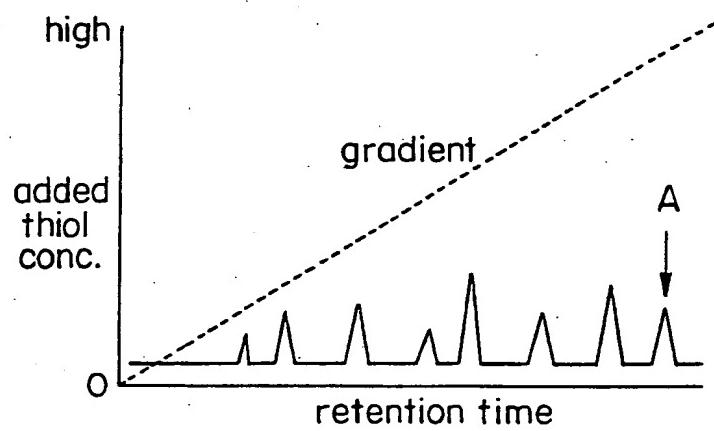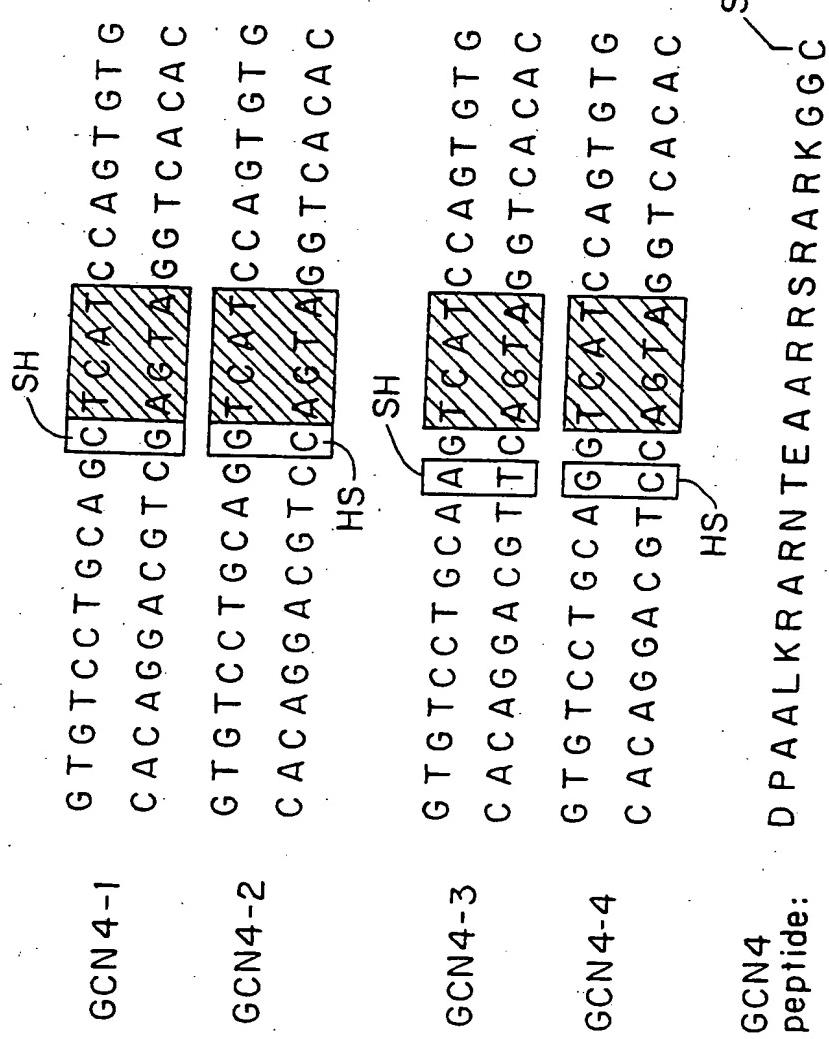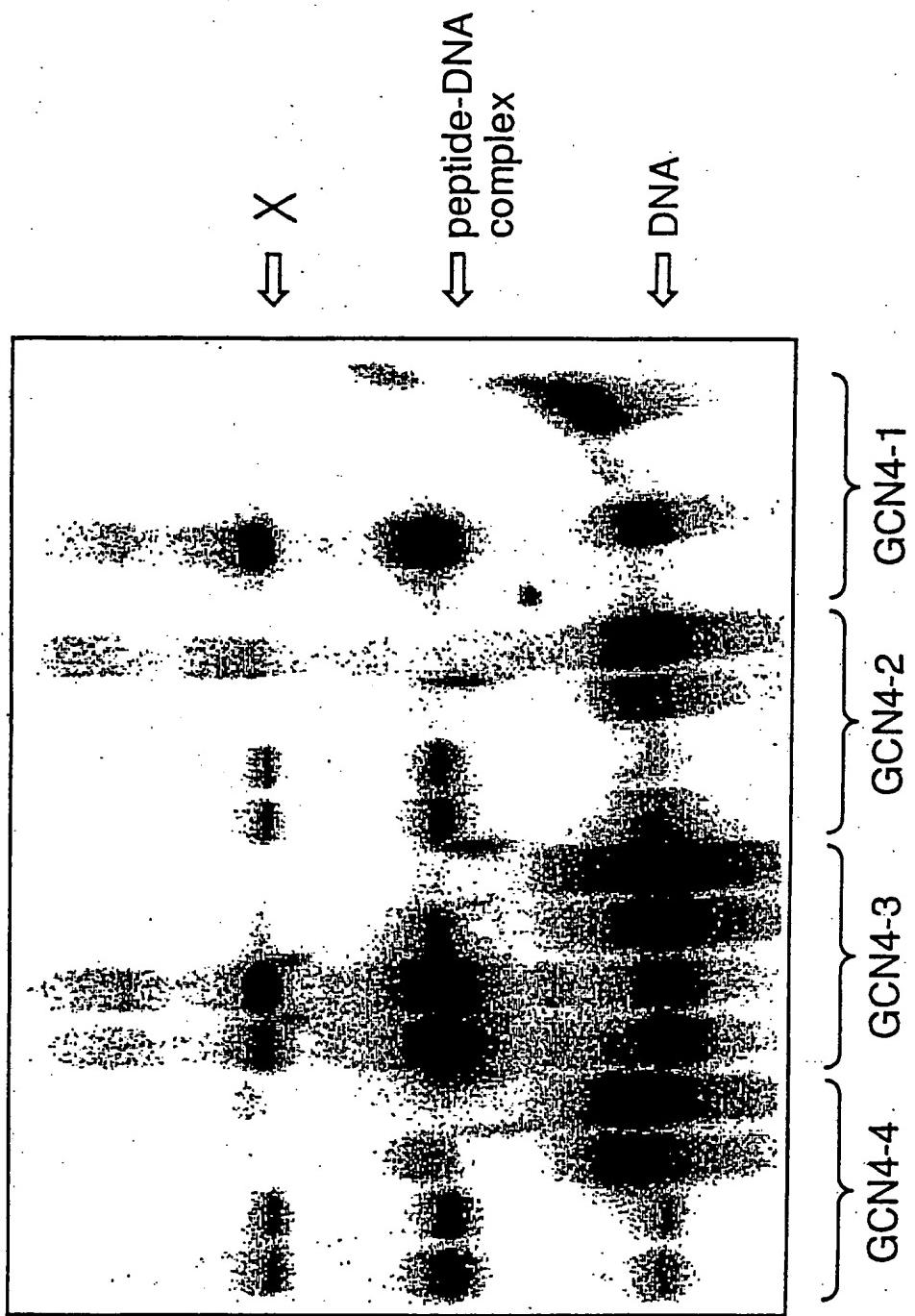
FIG. 1

FIG. 2

3 / 4

GCN4-1

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GTGTCCTGCAGC TCAT CCAGTGTG
CACAGGACGTCG AGTA GGTCACAC
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GCN4-2

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GTGTCCTGCAGG TCAT CCAGTGTG
CACAGGACGTCC AGTA GGTCACAC
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GCN4-3

```
GTGTCCTGCAAG TCAT CCAGTGTG
CACAGGACGTT CAGTA GGTCACAC
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GCN4-4

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GTGTCCTGCAGG TCAT CCAGTGTG
CACAGGACGTCC AGTA GGTCACAC
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GCN4 peptide:

DPAALKRARNTEAAARRSRARKGGC

FIGURE 3

FIG. 4

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C07K1/00;   C12Q1/68;   G01N33/68;   //C07K15/04

II. FIELDS SEARCHED

Minimum Documentation Searched⁷

Classification System	Classification Symbols
Int.Cl. 5	C12Q ; C07K ; G01N

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category°	Citation of Document,¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No.¹³
A	WO,A,8 910 931 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 16 November 1989 see page 4, line 6 - page 5, line 29 see page 19, line 25 - page 21, line 20; claims	1,3
A	WO,A,9 119 813 (THE UNIVERSITY OF COLORADO FOUNDATION) 26 December 1991 see page 13, line 21 - page 14, line 24 see page 63, line 6 - line 29	3
A	US,A,4 182 654 (G.P. ROYER) 8 January 1980 see column 2, line 36 - column 4, line 33; claims	1,3

° Special categories of cited documents :¹⁰

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
01 APRIL 1993	28. 04. 93

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	LUZZATTO E.R.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-8910931	16-11-89	US-A-	5010175	23-04-91
		AU-A-	3694289	29-11-89
		EP-A-	0417157	20-03-91
		JP-T-	3505254	14-11-91
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